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# Studies on nucleic acid reassociation kinetics: Rate of hybridization of excess RNA with DNA, compared to the rate of DNA renaturation*

**(bacteriophage φX174 nucleic acids/hydroxyapatite chromatography)**

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**ABSTRACT** **The rate of reaction of double-stranded replicative form (RF) [$^3$H]DNA of bacteriophage φX174 with excess (+)strand DNA and (+)strand RNA was measured by standard methods of hydroxyapatite chromatography. The reactions follow pseudo-first-order kinetics and the observed rate constant for the RNA·DNA reaction differs less than 25% from that of the DNA·DNA reaction. The pseudo-first-order rate constants are close to the value predicted on the basis of the second-order rate constant measured in the renaturation of the double-stranded φX RF [$^3$H]DNA.**

RNA excess hybridization reactions are widely used for the measurement of RNA sequence complexity. The rate at which such reactions proceed provides information regarding the prevalence in the total RNA of the hybridizing species as well as their complexity. In this paper we describe a measurement of the rate constant for the reaction between (+)strand RNA of bacteriophage φX174 present in excess and a double-stranded replicative form (RF) [$^3$H]DNA tracer. We show that this rate constant is similar or identical to that measured in an analogous reaction with excess (+)strand phage DNA. We conclude that the nucleation rates for the RNA·DNA and DNA·DNA reactions are comparable. A similar conclusion was reached by Hutton and Wetmur (1), who studied the formation of 1:1 hybrids of φX174 RNA and DNA by optical methods.

The RNA excess hybridization measurements described here were carried out with nucleic acid fragments 300–400 nucleotides (NT) long and were analyzed by hydroxyapatite chromatography. Corrections for disparity in tracer–driver lengths were thus unnecessary (effects of fragment length on tracer reactions analyzed by the hydroxyapatite method is the subject of a later paper of this series). The nucleic acid fragment lengths and the assay procedures (2) used in these experiments are similar to those now standard for studies of animal cell RNAs. Thus, we believe that the conclusions derived here will have wide applicability.

## MATERIALS AND METHODS

**Preparation of φX174 DNAs.** Unlabeled and $^{14}$C-labeled mature phage (+)strand DNA were the kind gifts of Lloyd H. Smith and Amy S. Lee, respectively. The nucleic acids were prepared essentially according to Razin *et al.* (3) and Lee and Sinsheimer (4). Unlabeled RF DNA was a gift of Lloyd Smith and was prepared according to Smith *et al.* (5). RF [$^3$H]DNA was prepared according to Johnson and Sinsheimer (6) and was a kind gift of Paul Johnson.

**Shearing and Determination of DNA Fragment Size.** φX DNAs were sheared to 300 NT single-strand length by two or three passages through a press at 50,000 pounds/inch$^2$ (340 MPa) in 0.2 M $Na^+$ (7). The DNAs were sized at about 300 NT (weight average) by alkaline sucrose gradient centrifugation, using internal or parallel DNA size standards previously sized by electron microscopy (8). We estimate the uncertainty of these determinations to be approximately ±10%.

**Synthesis of [$^{32}$P]RNA.** [$^{32}$P]RNA was synthesized from φX RFI DNA using α-$^{32}$P-labeled triphosphates as described by Smith and Sinsheimer (9). This RNA was a kind gift of Lloyd Smith. As detailed by Smith and Sinsheimer, 98–99% of the product is asymmetric, hybridizing to φX RF DNA but not to mature phage DNA. It is thus a (+)strand RNA. Thirty to thirty-five percent of the product is of discrete sizes ranging from 0.9 to 3 times unit length. The remainder is heterogeneous in length, the majority falling between 1 and 3 times unit length (9). After DNase treatment and deproteinization, the size of the [$^{32}$P]RNA was measured (see below) at 1600 ± 100 NT.

**Limited Hydrolysis of [$^{32}$P]RNA and Determination of RNA Fragment Size.** Limited alkaline hydrolysis of RNA was performed as described by Greenberg and Perry (10). φX [$^{32}$P]RNA in 1 mM ammonium acetate (pH 7) was mixed with an equal volume of 2 M NaOH at 22° for 55 sec and was then neutralized with 3 M acetic acid. The RNA was sized at 360 ± 20 NT by treatment with formaldehyde and centrifugation through formaldehyde-containing sucrose gradients (8), using sea urchin rRNAs and yeast 4S RNA as internal length markers.

**Reassociation of Nucleic Acids and Hydroxyapatite Chromatography.** Nucleic acids were denatured by heating for 1 min at 98° and then incubating at 60° in 0.12 M phosphate buffer containing 0.2% sodium dodecyl sulfate, or at 67° in 0.41 M phosphate buffer with 0.2% sodium dodecyl sulfate. Phosphate buffer is an equimolar mixture of basic and dibasic sodium phosphate, pH 6.8. Reassociation is expressed in terms of $C_0t$ (moles NT liter$^{-1}$ sec) corrected for acceleration in reassociation rate in buffers containing greater than 0.18 M $Na^+$ (7). To assay for hybridization reaction the mixtures were placed over hydroxyapatite columns at 60° in 0.12 M phosphate buffer, 0.2% sodium dodecyl sulfate. Duplexes that bound to the column were eluted at 98° in the same buffer. Radioactivity was determined by scintillation counting.

**Data Reduction.** Second-order and pseudo-first-order rate constants, and the most likely initial and terminal values for the reactions, were derived from the data by least squares analysis with the aid of a computer. A modification of the program published by Britten *et al.* (7) was utilized (16). The error estimates shown are internal and represent values derived from

Abbreviations: φX, bacteriophage φX174; NT, nucleotides; RF, replicative form; $C_0t$, mol of nucleotides per liter × sec.

* This is paper III in a series. Paper II is ref. 15.

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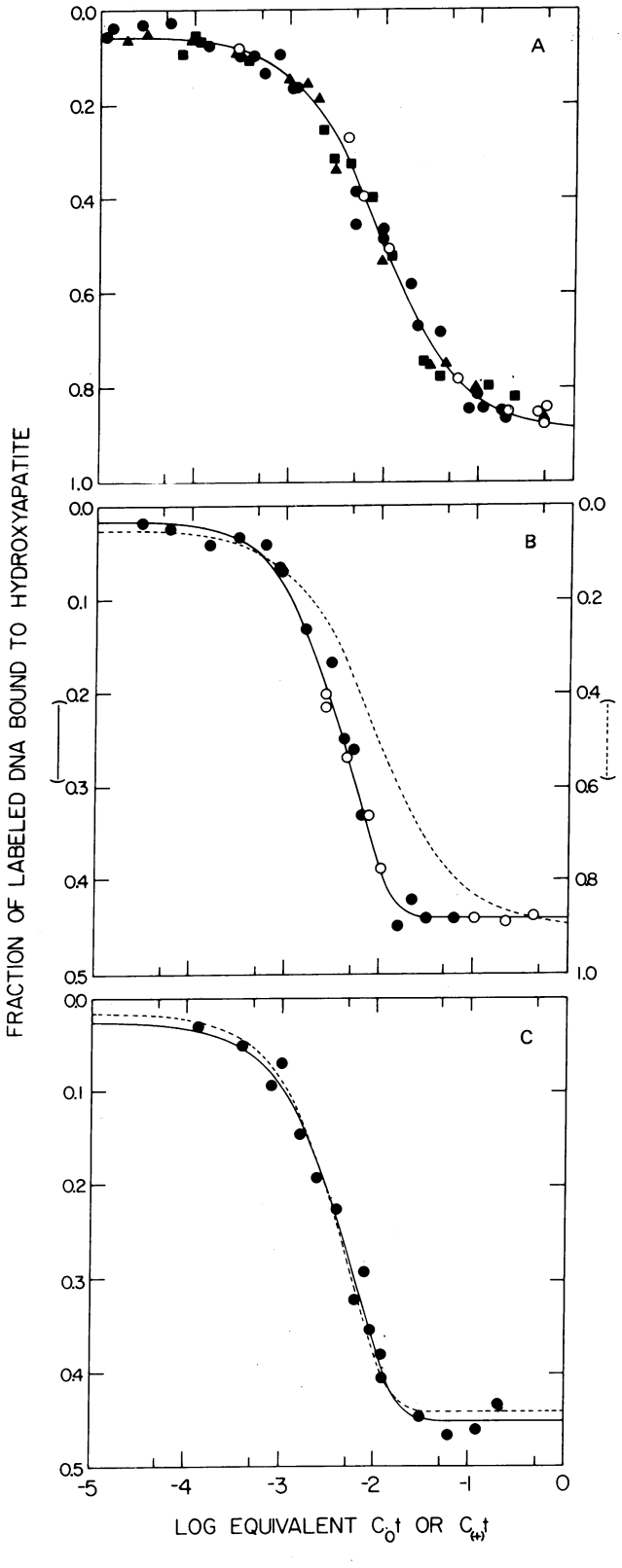


FIG. 1. Reassociation of 300 NT $\phi$X RF [$^3$H]DNA with $\phi$X DNAs and RNA. (*A*) Renaturation of RF [$^3$H]DNA. The renaturation of three separate preparations of RF [$^3$H]DNA is shown by solid symbols. Open circles indicate the reassociation of 300 NT (+)strand [$^{14}$C]DNA with a 180-fold excess of 300 NT unlabeled RF DNA. The solid line shows the best second-order function fit to the pooled data (Eq. 1). The solution shows that 84 ± 3% of the DNA reacts with an observed second-order rate constant ($k_{so}$) of 113 ± 14 liters mol$^{-1}$ sec$^{-1}$.

(*B*) Pseudo-first-order reassociation of RF [$^3$H]DNA with excess (+)strand DNA. RF [$^3$H]DNA was reassociated with a 170-fold mass excess of unlabeled (○) or a 17-fold mass excess of $^{14}$C-labeled (●) 300 NT (+)strand DNA. The solid curve represents the best least squares solution for the pooled data according to Eq. 2 with 43 ± 1% of the RF [$^3$H]DNA reacting with an observed rate constant ($k_{pfo}$) of 196 ± 14 liters mol$^{-1}$ sec$^{-1}$. For comparison (broken curve) the second-order renaturation of the RF [$^3$H]DNA is also reproduced from *A*.

(*C*) Pseudo-first-order reaction of RF [$^3$H]DNA with a 40-fold excess of 360 NT (+)strand [$^{32}$P]RNA. The solid curve shows the best solution for these data of the pseudo-first-order function described by Eq. 2, where $C_{(+)}$ now equals the starting and final concentration of (+)strand RNA; 42 ± 1% of the RF [$^3$H]DNA hybridizes. The least squares solution for the pseudo-first-order rate constant is 169 ± 16 liters mol$^{-1}$ sec$^{-1}$. The reaction of the RF [$^3$H]DNA with excess (+)strand DNA is reproduced from *B* in this figure as a broken line.

the least squares solutions. These error estimates reflect the scatter in the data, and not any possible systematic errors.

## RESULTS

### Second-order renaturation kinetics of $\phi$X174 RF DNA

The double-stranded RF [$^3$H]DNA was sheared to about 300 NT and allowed to renature under standard conditions. Fig. 1*A* illustrates the data obtained from several independent preparations of sheared RF [$^3$H]DNA. These behaved identically, with about 84% of the tracer participating in the reaction in each case. A few percent (4–5%) of the [$^3$H]DNA bound to hydroxyapatite at $C_0t$ 0, and the remaining 10–12% that appears not to react probably consists of fragments too short to form stable duplex structures. Also shown is a "slave" reaction in which the driver is *unlabeled* RF DNA and the tracer or "slave" is (+)strand [$^{14}$C]DNA of the same length. As expected, the renaturation of the [$^{14}$C]DNA follows the same kinetics as the self reaction of the RF [$^3$H]DNA. The least squares solution shown in Fig. 1*A* uses a function describing a second-order reaction. The form applied here is that introduced by Britten and Kohne (11):

$$\frac{C}{C_0} = \frac{1}{1 + k_{so}C_0t} \qquad [1]$$

in which C is the concentration of DNA remaining unreacted in moles NT liter$^{-1}$ (as determined by its inability to bind to hydroxyapatite) at time t (sec); $C_0$ is the initial denatured DNA concentration; and $k_{so}$ is the observed second-order rate constant.

According to the least squares analysis shown in Fig. 1*A* the best estimate of $k_{so}$ is 113 ± 14 liters mol$^{-1}$ sec$^{-1}$. Because about 84% of the [$^3$H]DNA participates in the reaction, the rate constant for a 100% pure preparation ("$k_{so}$ pure") would be 135 liters mol$^{-1}$ sec$^{-1}$. The genome size of $\phi$X174 is 5374 NT pairs (12), and from this the rate constant expected can be calculated from previous results obtained under the same conditions with other DNAs of known complexity. Applying measurements made on *Escherichia coli* DNA (11, 13), we would predict a rate constant for $\phi$X174 RF DNA of about 159 liters mol$^{-1}$ sec$^{-1}$. The least squares value obtained from the analysis shown in Fig. 1*A* differs by only 15%.

A pseudo-first-order reaction is expected to occur when the RF DNA tracer is reacted with excess (+)strand DNA, because the latter cannot react with itself. The concentration of the reactable driver DNA sequences will remain constant throughout the reaction and will determine the rate of reaction of the tracer. We let $C_{RF}$ represent the concentration of RF [$^3$H]DNA remaining unreacted at time *t*; $C_{RF_0}$ represents the

Table 1. Rate constants for second-order and pseudo-first-order reactions of 300 NT $\phi$X174 [$^3$H]DNA fragments*

| | |
|---|---|
| **Second-order renaturation** | |
| Observed $k_{so}$ | 113 ± 14 |
| Fraction of tracer reacting | 0.84 ± 0.03 |
| $k_{so}$ pure | 135 ± 17 |
| **Pseudo-first-order reactions with excess 300 NT (+)strand DNA** | |
| Observed $k_{pfo}$ | 196 ± 14 |
| Fraction of tracer reacting | 0.43 ± 0.01 |
| Estimated fraction of (+)strand DNA capable of reacting† | 0.85 ± 0.05 |
| Estimated $k_{pfo}$ pure | 231 ± 21 |
| Theoretical $k_{pfo}$ pure‡ | 270 ± 34 |
| Estimated $k_{pfo}$ pure / Theoretical $k_{pfo}$ pure | 0.85 ± 0.13 |

* Data are from Fig. 1*A* and *B*. All rate constants are in units of liter mol$^{-1}$ sec$^{-1}$.

† This value is estimated from the extent of reaction of 300 NT (+) strand [$^{14}$C]DNA with unlabeled RF DNA, and the extent of renaturation of similarly sized RF [$^3$H]DNAs shown in Fig. 1*A*. The presumption here is that the 14–15% single-stranded DNA observed in all these reactions at termination is due to the presence of very short fragments in the 300 NT (weight average) preparations, and that because the unlabeled (+)strand driver also has a 300 NT weight average fragment length, 15% of it will similarly be unreactive at the criterion applied.

‡ Theoretical $k_{pfo}$ pure = 2 × $k_{so}$ pure (see *text*).

initial concentration of denatured RF [$^3$H]DNA; $C_{(+)}$ represents the initial (and final) concentration of (+)strand driver DNA; and $k_{pfo}$ is the observed pseudo-first-order rate constant. Thus,

$$\frac{C_{RF}}{C_{RF_0}} = e^{-k_{pfo}C_{(+)}t}. \qquad [2]$$

This reaction is shown in Fig. 1*B*. In Fig. 1*B* $C_{RF}/C_{RF_0}$ is plotted against (+)strand DNA concentration × time, or $C_{(+)}t$. The solid line portrays the best fit of the function described by Eq. 2 to the data, according to least squares analysis. About 43% of the double-stranded RF tracer reacts, out of a maximum possible 50%. The fraction of the tracer reacting is 86% of the maximum possible, and thus is similar to that obtained in Fig. 1*A* for the second-order reaction.

The rate constants and fractions of tracer reacting in the experiments shown in Fig. 1*A* and *B* are listed in Table 1. The observed $k_{pfo}$ is of course smaller than the $k_{pfo}$ that would have been obtained had the driver (+)strand been 100% reactable. We have found that only 85% of the 300 NT RF DNA tracer reacts at the criterion applied here, and the same is expected of the 300 NT (+)strand driver (see second footnote of Table 1). If the (+)strand driver DNA also reacts to an extent of about 85%, we would obtain a "$k_{pfo}$ pure" of about 231 liters mol$^{-1}$ sec$^{-1}$ for the pseudo-first-order reaction. This would seem the most reasonable estimate from the data, but in any case, the purity correction affects the rate constant by only 15%.

We now compare the values of $k_{pfo}$ and $k_{so}$. When only the complementary strand is present the concentration of each single-stranded element of sequence is double the concentration for both strands. Therefore, for a nucleic acid of any particular complexity, $k_{pfo}$ should be about twice as high as $k_{so}$. In Table 1 the "theoretical" $k_{pfo}$ pure is obtained by doubling the measured $k_{so}$ pure, and is compared to the best calculated value for $k_{pfo}$ pure. It can be seen that the best calculated $k_{pfo}$ pure differs by only one standard deviation from the "theoretical" value. There are other various possible errors in the two rate determinations, and we have only estimated the reactivity of the 300 NT (+)strand driver. Thus, we consider the two numbers are equal within error and that they probably do not differ by more than 15–20%.

Table 2. Comparison of the pseudo-first-order kinetics of the reaction of 300 NT RF [$^3$H]DNA with excess 300 NT (+)strand DNA and with excess 360 NT (+)strand [$^{32}$P]RNA*

| | |
|---|---|
| **Reaction with 360 NT (+)strand [$^{32}$P]RNA** | |
| Observed $k_{pfo}$ | 169 ± 16 |
| Fraction of tracer reacting | 0.42 ± 0.01 |
| Estimated fraction of (+)strand [$^{32}$P]RNA capable of reacting† | 0.85 ± 0.5 |
| Estimated $k_{pfo}$ pure | 199 ± 22 |
| **Reaction with 300 NT (+)strand DNA‡** | |
| Observed $k_{pfo}$ | 196 ± 14 |
| Estimated $k_{pfo}$ pure | 231 ± 21 |
| **Comparison of $k_{pfo}$ for RNA reaction with $k_{pfo}$ for DNA reaction** | |
| Observed $k_{pfo}$ (RNA) / Observed $k_{pfo}$ (DNA) | 0.86 ± 0.10 |
| Estimated $k_{pfo}$ pure (RNA) / Estimated $k_{pfo}$ pure (DNA) | 0.86 ± 0.12 |

* Data are from Fig. 1*B* and *C*. All rate constants are in units of liter mol$^{-1}$ sec$^{-1}$.

† Reactability of this (+)strand [$^{32}$P]RNA driver was estimated from separate reactions with excess RF DNA (unpublished data).

‡ Data from Table 1.

## Pseudo-first-order reaction of RF [$^3$H]DNA tracer with excess (+)strand RNA

In Fig. 1*C* is displayed the kinetics of a reaction that is exactly analogous to that shown in Fig. 1*B* except that the (+)strand driver nucleic acid is [$^{32}$P]RNA rather than the mature phage DNA. The [$^{32}$P]RNA was prepared *in vitro* with RNA polymerase by Lloyd Smith, as described in *Materials and Methods*. When synthesized, it consisted mainly of greater than unit length transcripts (9). Thus, the RNA is of the same complexity as the $\phi$X genome, and should represent all regions of the genome to more or less equal extents. Smith and Sinsheimer (9) showed that the transcription product is 98–99% (+)strand. For the experiments described here, the RNA fragment length was reduced to a weight average size of about 360 NT by limited alkaline hydrolysis (see *Materials and Methods*).

Fig. 1*C* shows that the kinetics of the (+)strand-[$^{32}$P]RNA-driven reaction and the rate of the (+)strand-DNA-driven reaction are almost indistinguishable. When driven by the (+)strand RNA, about 42% of the tracer reacts, or about 84% of the maximum possible amount. The curve shown in Fig. 1*C* is the least squares solution to the function described by Eq. 2, where $C_{(+)}$ is now taken as the concentration of (+)strand RNA. The observed pseudo-first-order rate constant $k_{pfo}$ for this RNA·DNA hybridization reaction is 169 ± 16 liters mol$^{-1}$ sec$^{-1}$, not significantly different from the observed $k_{pfo}$ for the DNA-driven reaction, i.e., 196 ± 14 liters mol$^{-1}$ sec$^{-1}$. A detailed comparison of these rate constants is shown in Table 2.

Here it can be seen that the rate constant for the RNA excess reaction is 86% of the rate constant for the DNA excess reaction. The same conclusion is obtained whether the comparison is made between the observed $k_{pfo}$ values or the calculated $k_{pfo}$ pure values.

It is clear from these measurements that there is no significant difference in the rate between RNA·DNA and DNA·DNA pseudo-first-order reactions.

## DISCUSSION

Several minor uncertainties affect the comparison between the RNA excess and DNA excess pseudo-first-order rate constants. These include the errors on the determination of the rate constants, indicated by the standard deviations listed in Tables 1 and 2; possible errors in the determinations of the nucleic acid fragment lengths; and incomplete knowledge of the reactivities of the single-stranded driver nucleic acids. However, it is clear that none of these sources of uncertainty can produce important changes in the parameters measured. Thus, the second-order and pseudo-first-order rate constants obtained in this work are all close to expectation for a genome of the complexity of $\phi$X174. We conclude from the comparison summarized in Table 2 that the rate constant for the RNA-driven pseudo-first-order reaction is essentially the same as that for the DNA-driven pseudo-first-order reaction. A conservative statement would be that in RNA excess the nucleation rate for RNA·DNA hybridization is 75–100% of the nucleation rate for the DNA·DNA reaction.

This result agrees with a previous determination of Hutton and Wetmur (1), who also studied the reassociation of $\phi$X174 nucleic acids. The reactions employed by these authors differed from ours in that they measured the rate of formation of hybrids in 1:1 mixtures of (−)strand RNA and (+)strand DNA. It follows that RNA·DNA and DNA·DNA nucleation rates are approximately equivalent (under standard aqueous conditions), both in the reaction of stoichiometric quantities of complementary RNA and DNA strands and in the reaction of a double-stranded DNA tracer with a large excess of (+)strand RNA. This is as expected, because in RNA excess the (+)strand of the tracer is present in too low a concentration to compete with the excess (+)strand RNA. However, when excess DNA is reacted with a single-stranded RNA tracer a strikingly different result is attained. Under these conditions the RNA·DNA hybridization rate is observed to be only 15–30% of the DNA renaturation rate. This statement depends on an extensive series of reactions carried out with *E. coli* DNA and RNA, $\phi$X174 DNA and RNA, and ColE1 plasmid DNA and RNA, and also on kinetic measurements of the rate of hybridization of single-copy sea urchin DNA with mRNA tracers (8). DNA excess hybridization reactions with RNA will be discussed in detail in a later paper in this series. The fact that DNA excess and RNA excess hybridization behave differently accounts for much of the conflicting data on the rate of hybridization as compared to DNA renaturation to be found in the literature (e.g., refs. 1 and 14).

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